Trp promotor and a SD sequence. The resulting vector, pZRhi-1 (Figure 2) was used to express the hGH-mini-proinsulin fusion protein.

5.2. EXPRESSION OF hGH-MINI-PROINSULIN FUSION PROTEIN

The hGH-mini-proinsulin fusion protein expression vectors were transformed into *E.coli* strain RR1 or K12 W3110. The transformed *E.coli* cells were cultured in M9-CA media in the presence of trace elements. The expression level of the hGH-mini-proinsulin fusion proteins was determined in both shaking flask and fermentor. In both cases, a high level expression, amounting to 20-25% of total *E.coli* proteins, was observed.

5.3. REFOLDING OF THE hGH-MINI-PROINSULIN FUSION PROTEIN

The hGH-mini-proinsulin fusion proteins were expressed as an insoluble form termed "inclusion bodies". To release inclusion bodies, the *E coli*. cells were disrupted by high pressure homogenizer at 800 bar. The cell debris and soluble *E.coli* proteins were removed by a centrifugation at 10,000 g. The inclusion body pellets containing the hGH-mini-proinsulin fusion proteins were washed 3 times with water. The resulting inclusion body pellets, in which the hGH-mini-proinsulin fusion proteins was about 90% pure, was used as starting material for folding. The inclusion body was dissolved in 8 M urea, pH 10.4, at a hGH-mini-proinsulin fusion protein concentration of 20-30 mg/ml in the presence of 2 to 6 mM -mercaptoethanol. The insoluble material was removed by centrifugation. The supernatant was diluted 10 fold by low concentration urea to reach a final concentration of urea from 3 to 6 M, pH 9 to 10, and of -mercaptoethanol from 0.2 to 0.6 mM. The routine folding was carried out at 4°C at a urea concentration of 3.2 M, pH 9.3. The folding process was monitored by a C4 reverse phase HPLC with a 30-47% acetonitrile gradient in 0.1% phosphate buffer. Under such chromatographic conditions, the retention time of the correctly folded hGH-mini-proinsulin was around 23 min. The folding can be finished within 24 hr. The refolding yield was about 70%.

The folding mixture was fractionated by a 100K ultrafiltration. The correctly folded hGH-mini-proinsulin fusion proteins found in the filtrate fractions were concentrated by a 10 K ultrafiltration system. Urea was removed with water at pH 3.5. The yield of the ultrafiltration steps was over 85%.

5.4. TRYPTIC CLEAVAGE OF CORRECTLY REFOLDED hGH-MINI-PROINSULIN

For tryptic cleavage, the concentration of the correctly folded hGH-mini-proinsulin

fusion protein was present from about 10-12 mg/ml, preferably at 10 mg/ml. The ratio between trypsin and the hGH-mini-proinsulin fusion protein was ranged from about 1:60 to about 1:250, preferably at 1:100. pH was maintained from about 10 to about 11, preferably at 10.8. The cleavage was allowed to proceed at 4°C from about 1 to about 5 hr, preferably carried out for about 3.5 hr. The cleavage reaction was stopped by adjusting pH to 3.5 with phosphate buffer. Reverse phase HPLC analysis indicated that the yield for this cleavage step was more than 95%. At pH above 10, trypsin acts on the following Arg residues: the Arg residue between human mini-proinsulin B and A chains, the Arg residue between the hGH fragment and the mini-proinsulin fragment, the Arg residues within the hGH fragment. The trypsin digestion yielded several small pieces from the hGH fragment, and a human insulin with a extra Arg at C-terminus of B chain, which is termed as Arg(B31)-human-insulin. The Arg (B22) residue of human insulin was not cleaved under the above conditions, presumably due to the hinderance by three dimension structure.

The Arg(B31)-human-insulin was purified by cation exchange chromatographies using NaCl as eluent in the presence of 10 mM citrate buffer. The purified Arg(B31)-human-insulin was more than 90% pure.

5.5. CONVERSION OF ARG(B31)-HUMAN-INSULIN TO HUMAN INSULIN

The Arg(B31) at the C-terminus of the Arg(B31)-human-insulin was removed by carboxypeptidase B. The concentration of the correctly folded Arg(B31)-human-insulin was present at about 10 mg/ml. The ratio between carboxypeptidase B and the Arg(B31)-human-insulin was maintained at about 1:1000. The cleavage was allowed to proceed at 37°C for about 1 hr in 50 mM Tris-HCl buffer, pH 8.0. The cleavage reaction was stopped by adjusting pH to 3.5 with phosphate buffer. The yield of human insulin was more than 99%.

5.6. PURIFICATION OF HUMAN INSULIN

Human insulin produced from the carboxypeptidase B digestion was loaded onto a C8 reverse phase HPLC column that has been equilibrated with 0.1% phosphate buffer, pH 3.0. Human insulin was eluted by an acetonitrile gradient from 17% to 35% in 0.1% phosphate buffer, pH 3.0. Insulin fractions were pooled and acetic acid was added to reach a concentration of 0.125 to 0.2 M, pH 6.0. Insulin thus produced was crystallized at 4°C. The purity of the human insulin was over 99%.

5.7. CHARACTERIZATION OF THE PURIFIED HUMAN INSULIN

The first 15 N-terminal amino acids of the purified human insulin and the WHO standard human insulin were determined by standard Edman degradation. The N-terminal sequences of both A and B chains of the purified human insulin are identical to that of the WHO standard human insulin.

The molecular weight of the purified human insulin and that of the WHO standard human insulin was determined by VG Platform mass spectrometry analysis. Both samples gave a M/Z of 5807.7.

Both the purified human insulin and the WHO standard human insulin were digested with V8 protease. The digested fragments were analyzed by C18 reverse phase HPLC. Both samples gave identical peptide mapping pattern (See Thim et al., Genetics and Molecular Biology of Industrial Microorganisms, Hershberger et al., Ed. American Society for Microbiology, 1989, p322-328).

The present invention is not to be limited in scope by the microorganism deposited or the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.